Molecular and Cellular Endocrinology 251 (2006) 33–41

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ERK3 associates with MAP2 and is involved in glucose-induced insulin secretion

Gabriel F. Anhê [a], Andréa S. Torrão [a], Tatiane C.A. Nogueira [a], Luciana C. Caperuto [a], Maria E.C. Amaral [b], Mayrin C. Medina [a], Anna K. Azevedo-Martins [a], Angelo R. Carpinelli [a], Carla R.O. Carvalho [a], Rui Curi [a], Antonio C. Boschero [b], Silvana Bordin [a,*]

[a] *Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo (USP), 05508-900 São Paulo, Brazil*
[b] *Department of Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), 13083-970 Campinas, SP, Brazil*

Received 4 January 2006; accepted 14 February 2006

Summary

The adaptation of pancreatic islets to pregnancy includes increased β cell proliferation, expansion of islet mass, and increased insulin synthesis and secretion. Most of these adaptations are induced by prolactin (PRL). We have previously described that in vitro PRL treatment increases ERK3 expression in isolated rat pancreatic islets. This study shows that ERK3 is also upregulated during pregnancy. Islets from pregnant rats treated with antisense oligonucleotide targeted to the PRL receptor displayed a significant reduction in ERK3 expression. Immunohistochemical double-staining showed that ERK3 expression is restricted to pancreatic β cells. Transfection with antisense oligonucleotide targeted to ERK3 abolished the insulin secretion stimulated by glucose in rat islets and by PMA in RINm5F cells. Therefore, we examined the participation of ERK3 in the activation of a cellular target involved in secretory events, the microtubule associated protein MAP2. PMA induced ERK3 phosphorylation that was companied by an increase in ERK3/MAP2 association and MAP2 phosphorylation. These observations provide evidence that ERK3 is involved in the regulation of stimulus-secretion coupling in pancreatic β cells.

Keywords: Pancreatic islets; Insulin secretion; ERK3; MAP2

1. Introduction

The family of serine/threonine kinases known as extracellular signal-regulated kinases (ERKs) is composed by a large number of ubiquitous proteins being activated by a variety of hormones and growth factors (Pearson et al., 2001). In general, cell stimulation induces a signaling cascade that leads to phosphorylation of MEK (MAPK/ERK kinase), which, in turn, activates ERK via tyrosine and threonine phosphorylation (Robinson et al., 2002). The p44/p42 MAPKs (ERK1/2) are expressed in pancreatic β cell and regulate gene transcription and cell differentiation (Lingohr et al., 2002). Glucose, the main insulin secretagogue, induces phosphorylation of cytoplasmic proteins involved in exocytosis of insulin-containing granules such as synapsin I by activating the ERK1/2 cascade (Longuet et al., 2005).

Prolactin (PRL) upregulates islet function by increasing (1) insulin synthesis; (2) glucose uptake and metabolism; and (3) glucose-induced insulin release with a reduced glucose stimulation threshold (Sorenson et al., 1987a; Sorenson et al., 1987b; Shao et al., 2004). These changes induced by PRL are observed in vivo during early and late pregnancy (Weinhaus et al., 1996). A cDNA array approach was carried out in order to identify new proteins potentially involved in the insulin secretory machinery. We have found that PRL treatment increases ERK3 mRNA and protein content in isolated rat pancreatic islets (Bordin et al., 2004). ERK3 is a distantly related member of the mitogen-activated protein (MAP) kinase superfamily (Pearson et al., 2001; Turgeon et al., 2002). ERK3 is described as a highly unstable protein that is constitutively degraded by the ubiquitin–proteasome pathway in proliferating cells (Coulombe et al., 2003). In the cytoplasm, ERK3 is

* Corresponding author. Tel.: +55 11 30917961; fax: +55 11 30917285.
E-mail address: sbordin@icb.usp.br (S. Bordin).

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doi:10.1016/j.mce.2006.02.012

activated by the conventional PKCβ (Sauma and Friedman, 1996) and targets downstream activation of MAPK-activated protein kinase-5 (MK5) (Seternes et al., 2004; Schumacher et al., 2004).

In this study evidence is presented that ERK3 plays an important role for the functioning of rat pancreatic islet. The following aspects were examined: (i) the regulation of ERK3 expression by PRL during pregnancy, (ii) the distribution of ERK3 among the cell types of the pancreatic islet, (iii) the involvement of ERK3 in the glucose-induced insulin secretion, and (iv) the activation of ERK3 by conventional PKC.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, fraction V), Ficoll, RPMI-1640 medium, collagenase type V, and the chemicals used in the experiments for immunoblotting were purchased from Sigma (St. Louis, MO, USA). Lipofectamine™ 2000 Transfection Reagent and OPTI-MEM® I Reduced Serum Medium were purchased from Invitrogen (Carlsbad, CA, USA). All plastics for cell culture were obtained from TPP (Trasadingen, Switzerland). Nitrocellulose membranes (Hybond N, 0.45 μm), protein A sepharose 6 MB, and the enhanced chemiluminescence reagent kit (ECL) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The apparatus for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA, USA). Antibodies against ERK3 (sc-156), MAP2 (sc-20172), and α-Tubulin (sc-8035) were from Santa Cruz Technology (Santa Cruz, CA, USA). The antibody against phospho-serine was from Chemicon (Temecula, CA, USA), the anti-ERK1/2 antibody was from Cell Signaling Technology (Beverly, MA, USA) and the anti-PRLR antibody (mouse IgG1 clone U5) was from Affinity Bioreagents (Golden, CO, USA). The PKC inhibitor GÖ-6976 and phorbol-12-myristate-13-acetate (PMA) were from Calbiochem (Darmstadt, Germany). The oligonucleotides for ERK3 knockdown were manufactured by Integrated DNA Technologies (IDT, Coralville, IA, USA).

2.2. Prolactin receptor PRLR phosphorthioate oligonucleotide treatment and islet isolation

Phosphorthioate-modified oligonucleotides were designed according to the Genbank accession M74152 from *Rattus novergicus* PRLR. We have already detailed the treatment, the sequence of the antisense oligo and its in vivo efficiency (Amaral et al., 2004). The experiments presented herein were performed with the same set of animals used in the previous study. Briefly, female Wistar rats were injected (i.p.) with sense (SO-PRLR) and antisense (ASO-PRLR) oligonucleotides, respectively, during the 16th, 17th, and 18th days of pregnancy. The rats were killed on the day 19 of pregnancy. For each set of experiments, the islets were isolated from six rats by collagenase digestion of pancreata and separated from pancreatic debris by centrifugation in Ficoll gradient. The experiments involving animals were carried out in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.3. RNA extraction and RT-PCR

Total RNA was extracted from approximately 400 islets using Trizol reagent (Invitrogen). Conventional RT-PCR analysis was performed as previously described (Bordin et al., 2004). The amplification products were run on a 1.5% agarose gel containing ethidium bromide, and the band intensities were determined by digital scanning followed by quantification using the Scion Image analysis software (Scion Corp., Frederick, MD, USA). The results were expressed as a ratio of the target genes to the housekeeping RPL37a. The primer sequences used for RT-PCR analysis with their respective melting point and lengths were as follows: ERK3 sense 5′TGTTGTGAGGTCAGGAAGGACG and antisense 5′TGTCTTGTGAGGGATTTGAGGG, 57 °C, 309 bp; RPL37a sense 5′CAAGAAGGTCGGGATCGTCG and antisense 5′ACCAGGCAAGTCTCAGGAGGTG, 57 °C, 209 bp.

2.4. Immunostaining

The animals used for immunolabeling experiments (virgin Wistar female rats) were perfused with saline and 2% paraformaldehyde in 0.1 M phosphate buffer (PB) under ketamine/xylazine anesthesia and their pancreas postfixed for 5–8 h. After cryoprotection with 30% sucrose in PB for 48 h, the pancreata were sectioned (18 μm) on a cryostat and the sections immediately collected in gelatin and chromoalumen-coated slides. The sections were incubated with a combination of a guinea-pig polyclonal anti-insulin antibody (A-0564 from Dako, Denmark) and a rabbit polyclonal anti-ERK3 antibody (sc-156) from Santa Cruz Technology, both diluted 1:200 in PB with 0.3% Triton X-100. Following three times of 10 min washes in PB, sections were incubated with a mixture of a donkey anti-guinea-pig IgG labeled with fluorescein isothiocyanate and a donkey anti-rabbit IgG labeled with tetramethyl rhodamine isothiocyanate for 2 h (Jackson Laboratories, West Grove, PA, USA), both diluted at 1:50 in PB with 0.3% Triton X-100. Sections were then washed three times for 10 min in PB and coverslipped with glycerol-carbonate buffer. The material was analyzed under a fluorescence microscope and images captured using a digital camera. For control of immunostaining specificity, primary antibodies were replaced by normal sera.

2.5. RINm5F cell culture and incubations

RINm5F cells were cultured as previously described (Wilson et al., 1999). RPMI 1640-$NaHCO_3$ medium supplemented with 10% bovine fetal serum, 11.1 mM glucose, penicillin G (100 IU), and streptomycin (100 μg ml^{-1}), was used. For immunoprecipitation assay, cells were previously washed for 10 min at 37 °C with Krebs-bicarbonate buffer containing 2.8 mM glucose. After that, the cells were incubated in Krebs-bicarbonate buffer containing 2.8 mM glucose and GÖ-6976 (1 μM) for 2 h and then acutely stimulated with PMA (0.5 μM) for 20 min.

2.6. Pancreatic islets and RINm5F transient transfection with phosphorthioate oligonucleotide targeted to ERK3

Pancreatic islets from virgin Wistar rats and RINm5F cells were cultured in Opti-MEM containing 10 mM glucose plus a chimeric DNA-RNA 2′-*O*-methyl phosphorthioate oligonucleotide targeted to ERK3, previously mixed with Lipofectamine 2000 reagent. Islets and RINm5F cells were treated as follows: (1) with Lipofectamine 2000 reagent only (CTL), (2) with Lipofectamine 2000 mixed with a scrambled oligonucleotide (SO-ERK3), and (3) with Lipofectamine 2000 mixed with an antisense oligonucleotide (ASO-ERK3), and were incubated for 4 h in serum-deprived media. The medium was then supplemented with fetal bovine serum (10%) and cultured overnight. Islets and RINm5F cells were then used for insulin secretion assay and conventional immunoblotting against ERK3 to check the efficiency of the blockade. After transfection, RINm5F cells were also acutely stimulated with PMA (0.5 μM for 20 min) and used for MAP2 immunoprecipitation. The sequences of the SO-ERK3 and ASO-ERK3 were, respectively, 5′-mCmAmUmCGGCCCAATGCmCmGmAmC, and 5′-mGmCmCmUCCACAGCCCAmAmUmGmG, where 'm' denotes RNA 2′-*O*-methyl nucleotides.

2.7. Insulin secretion

Groups of five islets were first incubated for 30 min at 37 °C in Krebs-bicarbonate buffer containing 5.6 mM glucose and equilibrated with 95% O_2/5% CO_2, pH 7.4. The solution was then replaced by fresh Krebs-bicarbonate buffer and the islets were incubated for a further 30 min period with medium containing 5.6 or 11.1 mM glucose.

The RINm5F cells were seeded in 24-well plates and grown to reach near confluency. At the day of the experiment, the cells were first incubated for 30 min at 37 °C in Krebs-bicarbonate buffer containing 2.8 mM glucose and equilibrated with 95% O_2/5% CO_2, pH 7.4. The solution was then replaced